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Review

Quantitative methods for analyzing cell–cell adhesion in development

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ABSTRACT

During development cell–cell adhesion is not only crucial to maintain tissue morphogenesis and homeostasis, it also activates signalling pathways important for the regulation of different cellular processes including cell survival, gene expression, collective cell migration and differentiation. Importantly, gene mutations of adhesion receptors can cause developmental disorders and different diseases. Quantitative methods to measure cell adhesion are therefore necessary to understand how cells regulate cell–cell adhesion during development and how aberrations in cell–cell adhesion contribute to disease. Different *in vitro* adhesion assays have been developed in the past, but not all of them are suitable to study developmentally-related cell–cell adhesion processes, which usually requires working with low numbers of primary cells. In this review, we provide an overview of different *in vitro* techniques to study cell–cell adhesion during development, including a semi-quantitative cell flipping assay, and quantitative single-cell methods based on atomic force microscopy (AFM)-based single-cell force spectroscopy (SCFS) or dual micropipette aspiration (DPA). Furthermore, we review applications of Förster resonance energy transfer (FRET)-based molecular tension sensors to visualize intracellular mechanical forces acting on cell adhesion sites. Finally, we describe a recently introduced method to quantitate cell-generated forces directly in living tissues based on the deformation of oil microdroplets functionalized with adhesion receptor ligands. Together, these techniques provide a comprehensive toolbox to characterize different cell–cell adhesion phenomena during development.

Introduction

Cell–cell adhesion in development

During embryonic development the ability of cells to adhere to one another is fundamental for the assembly of a three-dimensional tissue and forms the basis for the formation of multicellular organisms. Cell–cell adhesion is not only important to simply keep cells together but also to organize them in complex tissues with diverse and distinctive patterns. A first significant demonstration of the importance of cell–cell adhesion for germ layer assembly was made by Townes and Holtfreter in the 1950s. Using dis- and re-association assays of amphibian embryonic cells and tissues, the authors demonstrated that randomly intermixed cells spontaneously self-organize to reconstitute the different germ layers. Interestingly, the rearranged tissues reflected the same arrangement as native tissues during normal embryonic development, whereby the ectoderm is located in the periphery, the endoderm is internal, and the mesoderm is arranged in the region between them (Barriga et al., 2013). Holtfreter termed this phenomenon as 'selective affinity', although the mechanism providing the driving force underlying these cell and tissue rearrangements was not clear defined. Later, Steinberg attributed this phenomenon to differential cell–cell adhesion processes and proposed the 'differential adhesion hypothesis' (DAH) (Foty and Steinberg, 2013; Steinberg, 1996, 2007). DAH defines that tissues behave like unmixable liquids with a given surface tension and that differences in tissue surface tension control cell segregation and tissue organization. Furthermore, Steinberg proposed that tissue surface tension scales with cell adhesion so that differences in cell adhesion among different cell types drives tissue segregation. DAH was verified experimentally both in the developing *Drosophila* retina and in cell culture (Foty and Steinberg, 2005; Hayashi and Carthew, 2004). Moreover, several studies demonstrate the importance of differential adhesion during morphogenesis, including *e.g.* cell sorting in the *Drosophila* imaginal wing disc (Chang et al., 2011; Dahmann and Basler, 2000), rhombomere domain boundary formation (Cooke et al., 2005), gastrulation movements (Maitre et al., 2012; Ninomiya et al., 2012; Shimizu et al., 2005) and neural crest migration (Mayor and Theveneau, 2013; McKeown et al., 2013). However, DAH has remained

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controversial and alternative hypotheses have been suggested to explain cell sorting. For instance, Harris proposed the differential surface contraction model (DSC) in which cell sorting is driven by differences in actomyosin-dependent cortical tension, rather than by cell–cell adhesion *per se* (Harris, 1976). Subsequently, the differential interfacial tension hypothesis (DITH) was introduced by Brodland which combines elements from both DAH and DSC theories (Brodland, 2002). The DITH postulates that cell rearrangements are controlled by interfacial tension, which largely depends on both cell adhesion and cell contraction. Taken together, it should be emphasised that a balance between cell–cell adhesion, cortical tension and cortical elasticity promote surface and interfacial tension as discussed in different studies (Farhadifar et al., 2007; Foty and Steinberg, 2013; Krieg et al., 2008a; Lecuit and Lenne, 2007; Paluch and Heisenberg, 2009), although the exact interplay between these mechanisms remains elusive.

Cadherins

A key element in the regulation of tissue morphogenesis is the formation, rearrangement and maintenance of physical cell–cell contacts mediated by different adhesion molecules and cell surface ligand and receptor systems (Yamada and Nelson, 2007). Several classes of adhesion molecules, including members of the immunoglobulin superfamily (Cunningham, 1995), selectins (Rosen and Bertozzi, 1994) and cadherins mediate cell–cell adhesion and control the physical interactions between cells. This review focuses primarily on methods to study adhesion mediated by cadherin receptors, one of the most comprehensively studied families of cell–cell adhesion receptors in a developmental context. Cadherins form a multigene family of Ca^{2+}-dependent glycoproteins promoting homotypic cell–cell adhesion in most animal species (Oda and Takeichi, 2011). Cadherins are particularly important for the dynamic regulation of adhesive contacts and they are therefore crucial for promoting diverse morphogenetic processes. Intense research into cadherin function started in the early 1980s, when Jacob and co-workers described the role of E-cadherin (uvomorulin) in blastomeres compaction of an early developing mouse embryo (Hyafil et al., 1981; Peyrieras et al., 1983).

Classical cadherins are transmembrane proteins that mediate cell–cell adhesion by forming intracellular bonds through interactions of their extracellular sub-domains on opposed cells (trans-orientation) by a mechanism called strand swapping (Posy et al., 2008; Zhang et al., 2009). The cytoplasmic domain of classical cadherins contains a β-catenin binding site, which dynamically links cadherins to the actin cytoskeleton via α-catenin (Drees et al., 2005; Pokutta et al., 2008). Anchoring of cadherins to the cytoskeleton is also promoted by recruitment of actin-binding proteins, such as epithelial protein lost in neoplasm (EPLIN) and vinculin (Abe and Takeichi, 2008; Alfandari et al., 2010). Importantly, the recruitment of actin-binding proteins induces remodelling of the underlying cortical cytoskeleton, with consequential changes in the mechanical properties of the cells (le Duc et al., 2010; Liu et al., 2010; Taguchi et al., 2011; Yonemura et al., 2010). In recent years several studies have focused on biophysical descriptions of cadherin function in cell–cell contact formation during morphogenesis, including aspects of interfacial tension, signalling to the actomyosin cytoskeleton and the mechanical coupling of contacting cells (Maitre and Heisenberg, 2013).

Apart from forming robust cell–cell contacts, cadherins mediate a number of intracellular signalling cascades that control cell proliferation (Kim et al., 2011; Nelson and Chen, 2003), cell polarity (Bosveld et al., 2012; Wang et al., 2010) and cell fate specification (Lorthongpanich et al., 2012; Stephenson et al., 2010). Moreover, cadherins modulate cell sorting, cell cortex tension, and promote cell migration of different cell types (Becker et al., 2012; Foty and Steinberg, 2004; Halbleib and Nelson, 2006; Maitre et al., 2012; Niessen et al., 2011; Takeichi, 1995). Dysregulation of cadherin adhesion and signaling function on the other hand leads to a broad variety of pathological defects including tumor invasion and metastasis (Berx and van Roy, 2009), inflammatory diseases (Hermiston and Gordon, 1995; Karayiannakis et al., 1998; Lee et al., 2007) or causes congenital defects in organogenesis (El-Amraoui and Petit, 2010).

During development different cell types migrate through the embryo and this requires constant modulation of cell–cell adhesion. One cell population that displays high motility during development is the neural crest (NC), a multipotent and highly motile cell population specific for vertebrates (Mayor and Theveneau, 2013). Importantly, NC cells (NCC) migrate collectively as a cohesive tissue. This recognized mode of migration also occurs in border cell migration in *Drosophila* and lateral line migration in zebrafish, as well as in wound healing and cancer metastasis (Friedl and Gilmour, 2009; Rorth, 2009). However, after a distinct time NCC progressively dissociate from the cell sheet and migrate as single cells until they find their final destination (Alfandari et al., 2010). Thus, cell–cell adhesion has to be precisely and continuously modulated during NCC migration. Interestingly, NCC not only display a similar migration behavior as invasive cancer cells, they also up-regulate a similar set of adhesion molecules, including cadherin-11 and N-cadherin (Tomita et al., 2000). NCC are therefore an excellent model system for investigating cell adhesion mechanisms underlying collective and single cell migration.

Another highly motile embryonic cell population are primordial germ cells (PGCs), which migrate as individual cells from the place where they are specified to the site of gonad formation (Richardson and Lehmann, 2010). Work in zebrafish provides evidence that E-cadherin mediated cell–cell adhesion is crucial for PGCs motility *in vivo* (Kardash et al., 2010). During migration PGCs form dynamic E-cadherin mediated contacts with neighboring somatic cells. PGCs then employ retrograde flow of actin-rich structures to exert pulling forces on these cadherin contacts, ultimately generating sufficient traction forces for the proper migration of these cells through the surrounding tissue (Kardash et al., 2010). Interestingly, a recent *in vitro* study using *Xenopus* PGCs demonstrates that pre-migratory PGCs exhibited stronger adhesion to somatic cells than migratory PGCs (Dzementsei et al., 2013). This observation is in direct correlation with the down-regulation of E-cadherin expression during PGC migration, which might contribute to the weakening of cell–cell adhesion contacts (Dzementsei et al., 2013). Thus, similar to NCC, cell–cell adhesion has to be precisely modulated for proper PGC migration.

In recent years advances in microscopy techniques and biophysical measurements have provided the possibility to identify biomechanical mechanisms underlying the formation and function of cell–cell contact, cell migration and tissue remodeling. Among these, different *in vitro* adhesion assays have been developed to characterize the adhesion strength between cells, which is generally measured by the ability of cells to remain attached to each when exposed to external forces. For instance, centrifugal assays in combination with radioactive cell labeling (Lotz et al., 1989; McClay et al., 1981) have been used to determine the formation kinetics of E-cadherin adhesion contacts in mouse fibroblasts (Angres et al., 1996), while shear flow assays are useful for studying adhesive interactions between endothelial cells and leukocytes (Kucik, 2009). Bulk adhesion assays offer the possibility to test a large number of cells, generating statistically relevant data within a short time frame. However, they determine the average behavior of cell populations and provide little information regarding the behavior of an individual cell (Chu et al., 2004). As a consequence, small differences in cell adhesion that are of potential biological significance are difficult to detect. For instance, adhesive

subpopulations arising from different functional states of individual cells cannot be identified. In addition, short contact times are difficult to control, while the shear forces of the buffer stream may be too weak to dislodge tightly adhering cells after longer attachment periods. Quantitative analysis of cell adhesion therefore requires single-cell techniques. Biophysical techniques to characterize cell adhesion on the level of single cells include atomic force microscopy (AFM)-based single-cell force spectroscopy (SCFS) (Benoit et al., 2000) and dual micropipette aspiration (Sung et al., 1986). These quantitative methods provide tools for obtaining a better understanding of how cells regulate cell–cell adhesion in the establishment and maintenance of multicellular organisms. In this review we will give an overview about how quantitative single-cell methods can be applied to investigate developmentally-regulated adhesion processes. Furthermore, we discuss how these experimentally more demanding single-cell techniques can be complemented by semi-quantitative adhesion assays providing an easier experimental approach for analyzing cell–cell adhesion. Furthermore, we discuss recent applications of Förster resonance energy transfer (FRET)-based molecular tension sensor strategies to measure the tension in the pN-range experienced by cadherin cytoplasmic domains at cell–cell contacts.

Quantitative methods for analyzing cell–cell adhesion

Flipping assay

An experimentally straightforward but semi-quantitative flipping assay to analyse cell–cell adhesion has recently been introduced (Carmona-Fontaine et al., 2011). In this experimental approach, one set of cells are first grown or explanted as monolayers on an extra cellular matrix (ECM) substrate. Subsequently, a second set of cells depleted for a specific adhesion protein by gene knockdown are dissociated, mixed with fluorescently-labelled control cells and co-seeded on the initial monolayer. After several minutes, the dish is rotated by 180°, held in an upside-down position, carefully shaken, and finally rotated back into the original position. From pictures taken before and after dish flipping, the percentage of knockdown and control cells adhering the cell monolayer support after substrate inversion is determined, which provides information regarding the scale of cell–cell adhesion mediated by the investigated receptor. Importantly, in this assay untreated control cells are tested in parallel with the manipulated cells, providing directly comparative results (Fig. 1). Compared to single-cell assays, the flipping assay is a comparatively fast and reliable way without the need of extensive laboratory equipment to investigate the function of different adhesion molecules in mediating cell–cell adhesion in different *in vitro* cell systems.

The usefulness of this technique for studying cell adhesion during development has been demonstrated for *Xenopus* cranial NCC (CNCC) (Carmona-Fontaine et al., 2011). Here, wild type CNCC cells were first explanted as monolayers on fibronectin, and control and N-cadherin morpholino-treated CNCC were then added. While the majority of CNC control cells remained adhered to the monolayer after inversion, a significant number of N-cadherin knockdown CNCC were lost after inversion, indicating reduced cell–cell adhesion between wild type and N-cadherin depleted CNCC. This experiment demonstrates the importance of N-cadherin in mediating cell–cell adhesion between CNCC.

Atomic force microscopy (AFM)-based single-cell force spectroscopy (SCFS)

Bulk adhesion assays are usually easy to perform and yield fundamental insight into adhesion processes mediated by different adhesion receptors during embryogenesis. However, these assays provide only averaged results regarding the relative adhesion strength of entire cell populations, without the possibility to quantitatively measure adhesion forces between individual cells. Quantitatively analyzing adhesive properties within different subpopulation requires the implementation of single-cell techniques. Single-cell techniques are also useful in experiments where bulk adhesion assays cannot be used because the number of cells available for adhesion measurements is limited. For instance, during embryonic development, the specific adhesive properties of different cell lineages play an important role in driving tissue formation but collectively probing adhesion of these cells is often difficult due to their relatively small number in the developing embryo. However, single-cell assays usually require the isolation of individual probe cells from their *in vivo* environment prior to measurement, and it is largely unknown how the sudden loss of biochemical and mechanical cues presented within intact embryonic tissues affect cell function.

AFM-based single-cell force spectroscopy (SCFS) is an ultrasensitive method to quantify cellular adhesion forces of individual cells under physiological conditions (Benoit et al., 2000; Franz and Taubenberger, 2012; Friedrichs et al., 2013). In SCFS adhesion measurements, a living cell is first attached to the apex of a tipless AFM cantilever (Fig. 2). To facilitate cell immobilization, cantilevers can be coated with different matrix proteins, such as fibronectin or laminin, or with sugar-binding lectins, which provides gentle cell attachment via glycosylated cell surface proteins (Benoit et al., 2000; Puech et al., 2006). Alternatively, living cells have been biotinylated and coupled to streptavidin-coated cantilevers (Panorchan et al., 2006). The cantilever-attached cell is then approached onto a second cell attached to a surface and cell–cell contact is maintained under constant contact force for a pre-defined dwell time. During subsequent cell retraction, cellular adhesion forces can be measured with high sensitivity from the degree of cantilever deflection with single-molecule resolution (Benoit et al., 2000; Panorchan et al., 2006). In contrast to other single-molecule experiments using recombinant proteins, in SCFS cellular receptors are tested in their natural molecular environment at the plasma membrane, which includes the potential action of associated transmembrane or intracellular proteins and possible linkage to the cytoskeleton. The ability to measure forces with high resolution over a wide range makes AFM furthermore a unique tool to study cellular adhesion forces from the single-molecule level to that of the entire cell (Franz and Puech, 2008). The piezo-driven cantilever positioning system of AFM also provides exquisite temporal and spatial control over the cell–cell interaction. Even for short contact times (<1 s), SCFS is well-suited to investigate initial events of cell adhesion. By varying the dwell time, progression from single-receptor to cooperative receptor binding can be monitored on the force level and correlated with the establishment of overall adhesion (Fichtner et al., 2014; Selhuber-Unkel et al., 2008; Taubenberger et al., 2007). The cantilever-attached cell can also be approached to map adhesive properties of different regions on tissue explants or cellular monolayers (Fierro et al., 2008).

SCFS experiments can be performed using a standard AFM setup with an incorporated light microscope. The light microscope facilitates cell immobilization on the cantilever and also permits the investigator to approach the probe cell accurately above a second cell or at different locations of a tissue. Furthermore, fluorescently-labelled cells, indicating cell type or knockdown manipulation, can be reliably identified. However, cells may deform strongly under an applied pulling force, including the extrusion of long plasma membrane tethers, and complete cell–cell separation after the formation of strong adhesion usually requires an extended pulling range in z-direction (Puech et al., 2006). This can be achieved either by using an AFM head with an extended pulling z-range or by incorporating a piezo-driven vertical pulling mechanism into the sample stage.

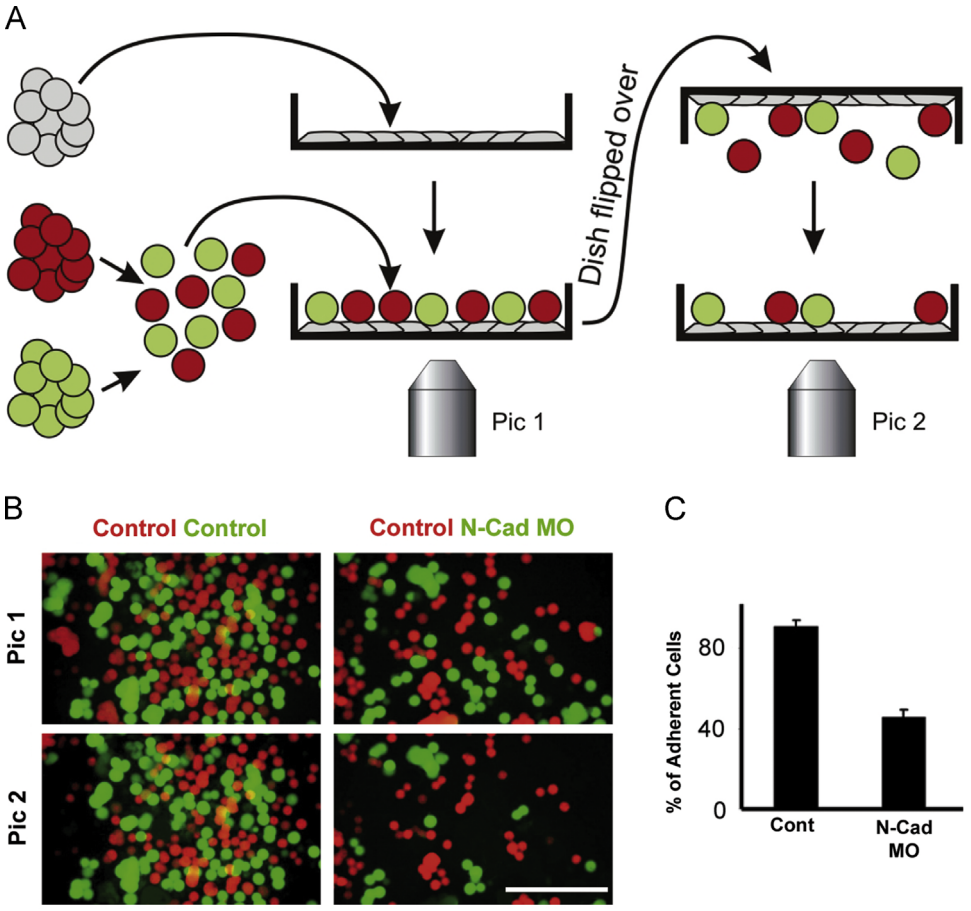

Fig. 1. Cell–cell adhesion flipping assay. (**A**) Wild type CNC cells were cultured as a monolayer on a fibronectin coated dish (gray cells). Control (red) and N-cadherin morphant (green) CNC cells were dissociated, mixed, and placed on the CNC monolayer. After several minutes, the dish was turned by 180°, shaken, and the number of labelled CNC cells remaining on the substrate was counted. For determining the number of adhering CNC cells on the monolayer, pictures were taken before (Pic 1) and after (Pic 2) the dish was inverted. (**B**) Control CNC cells remained adhering on the monolayer in the majority, whereas a significant number of N-cadherin deficient CNC cells were lost. Scale bar: 150 μm. (**C**) Statistical analyses of adhering CNC cells. Figure modified with permission from (Carmona-Fontaine et al., 2011). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

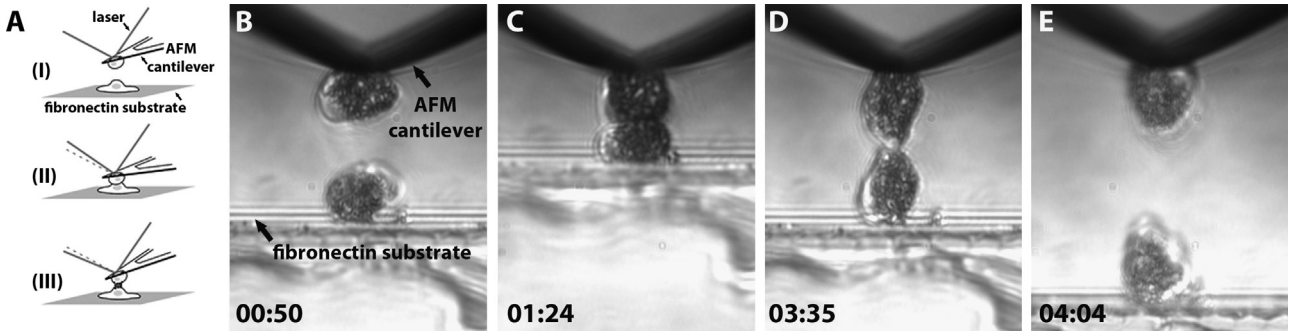

Fig. 2. AFM-based single-cell force spectroscopy. (A) Schematic depiction of an AFM-based SCFS experiment. A single cell immobilized on a functionalized cantilever is positioned above a second cell adhering to a fibronectin substrate (I). Both cells are then brought into contact for a defined contact time and with a preset contact force (II). The cells are subsequently separated, and the maximal separation force and the detachment work can be extracted from a simultaneously recorded force–distance-curve (III). (B) SCFS experiment using a mirror-based side view setup to visualize the cell couple in horizontal direction. A single CNCC cell immobilized on a concanavalin A-functionalized cantilever is positioned above a second CNCC cell attached to a fibronectin substrate. (B) The two cells are then brought together with a contact force of 1 nN. (C) After a contact time of 120 s, the cantilever is retracted until the cells separate (D).

In addition to quantitative information about overall cell adhesion, SCFS force–distance curves also contain a series of small rupture steps which provide information about the number and strength of the individual adhesive units mediating cell contact. Analyzing the step size of these rupture steps, which can represent the rupture of individual or small clusters of receptors, showed that individual E-cadherin receptors unbind sequentially in a zipper-like fashion, instead of simultaneously as larger groups of cross-linked receptors (Fichtner et al., 2014). At the same time, the number of force steps per force curve increases with contact time, suggesting that cadherin

contacts are reinforced primarily by increasing the number of receptors organized into a comparatively loosely associated array. Thus, analyzing SCFS force curves can provide additional information regarding the receptor unbinding mechanism in a cellular context.

In SCFS cell–cell separation occurs under non-equilibrium conditions and the separation forces are therefore rate-dependent (Benoit et al., 2000). Quantitative differences in cell adhesion strength can be obtained when cells are separated at the same speed or loading rate. Likewise, contact forces should be similar in comparative measurements, as higher impingement forces have been shown to lead to stronger adhesion (Panorchan et al., 2006).

So far mainly cell lines have been used in SCFS experiments, but recently this technique has been also been extended to test adhesion between different primary cell types isolated from developing embryos at specific developmental stages. Since cell isolation and attachment to the AFM and subsequent adhesion force measurements take some time (>1 h), testing single cells at specific stages requires careful experimental planning in these experiments. Furthermore, the identity of the tested cell type must be verified before cantilever attachment. For instance, to compare the adhesive properties of different germlayers during zebrafish gastrulation, ectoderm, mesoderm and endoderm progenitor cells were identified by specific lineage markers in gastrulating zebrafish embryos and tested in SCFS experiments (Krieg et al., 2008a). Measurements between two cells of the same type (homotypic interaction) revealed that at all contact times tested ectoderm cells show lower adhesion compared to their mesoderm and endoderm counterparts. Heterotopic adhesion levels were comparable to homotypic interaction between ectoderm cells, the least adhesive subpopulation. These results demonstrate that mesendoderm and endoderm progenitors are more cohesive than ectoderm cells. Interestingly, the degree of adhesion strength in all three cell types roughly correlated with the level of E-cadherin expression, indicating that E-cadherin is the main receptor responsible for the differential adhesion properties of progenitor cells. Despite these cell-type specific differences, however, E-cadherin-mediated differential adhesion alone is insufficient to explain the cell sorting behavior. Instead, differences in actomyosin-dependent cell-cortex tension are critical to drive progenitor cell sorting (Krieg et al., 2008a).

Establishing differential cell adhesion profiles of single cells by comparative SCFS

SCFS adhesion experiments provide quantitative information about receptor-mediated cell–cell adhesion strength. However, cells generally express different adhesion receptors simultaneously, making it difficult to dissect the individual contribution of a specific receptor type to overall cell adhesion. In particular, different cell types, *e.g.* NCC, are known to dynamically modulate the expression levels of different cadherin subtypes during development, and determining the scale and functional relevance of adhesion transmitted by a particular cadherin subtype at different developmental stage is difficult. Here, it can be advantageous to restrict adhesive interactions to a single type of cadherin receptor by conducting single-cell adhesion measurements on artificial adhesion substrates. For example, using artificial E-cadherin substrates in SCFS experiments, it could be shown that in zebrafish the cohesive properties of zebrafish ectoderm, mesoderm and endoderm progenitors scale specifically with the level of E-cadherin-mediated adhesion (Krieg et al., 2008a).

To retain the adhesive functionality of the cadherin molecule of choice in these surface assays, it should be coupled to the surface in its proper orientation and at defined density. This can be achieved for instance using a SNAP-tag-based surface immobilization protocol to covalently couple recombinant cadherin ectodomains to self-assembled monolayers (Engin et al., 2010) or supported lipid membranes (Korner et al., 2013). These cadherin surfaces provide a platform for reproducible single-cell and SCFS experiments (Fichtner et al., 2014) and can also be combined with microcontact printing to generate patterned cadherin surfaces. By selectively functionalizing different area on the substrate with different cadherin subtypes, multifunctional cadherin surfaces could then be used for differential SCFS adhesion measurements to directly compare the adhesion strength of a single cell to different cadherins (Dao et al., 2012, 2013). Sequentially approaching a single cell attached to an AFM cantilever to different cadherin functionalizations could then generate a comprehensive differential cadherin adhesion profile of the single probe cell at different stages of development.

Dual micropipette aspiration

Micropipette-based assays are an alternative method to quantitate cell–cell adhesion forces. In the dual micropipette assay (DPA), two micropipettes attached to a pressure control system each hold a single cell under gentle aspiration (Chu et al., 2004; Daoudi et al., 2004; Sung et al., 1986). The cells are then brought together for a defined contact time to initiate cell adhesion formation (Fig. 3). Subsequently, sufficiently high aspiration to stably hold the cell pair is applied to one of the micropipettes, while aspiration in the other pipette is increased in increments. After each pressure increase step, the pipettes are moved apart in an effort to separate the cells. This procedure is repeated until the aspiration level in the second micropipette is strong enough to separate the cell couple. The cell–cell separation force can be calculated from the aspiration pressure required to break the cell–cell interaction during micropipette retraction. When testing previously unassociated cells, the cell–cell contact time can be precisely adjusted and the influence of contact time on adhesion strength contact can be determined. Alternatively, pre-formed cell doublets can be separated using DPA, avoiding the need to pause for a defined contact time before cell separation, but in this case the contact history of the cell pair is undefined. The force resolution of the step-pressure DPA assay ($\sim$nN range) is lower than AFM-based SCFS ($\sim$pN range) and DPA does not resolve individual molecular interactions between cells. Nevertheless, DPA permits accurate quantification of overall adhesion forces between cells and is not limited at the upper force range (see below). If single-molecule force resolution is required, the biomembrane force probe (BFP) can be used, which is a variant of the dual micropipette assay using a tensed erythrocyte as a force sensor (Evans et al., 1995). The BFP is an ultrasensitive force measuring device and has yielded unique information into the molecular mechanisms underlying single receptor–ligand interactions, but it has been less widely used to study developmentally-relevant processes, which occur primarily on the cell and tissue scale.

DPA has been instrumental in quantitating cadherin-mediated adhesion in tissue culture systems (Chu et al., 2004, 2006; Martinez-Rico et al., 2005), but is also gaining popularity in studies aimed at elucidating developmentally-regulated cell adhesion. For instance, the role of tissue cohesion for the migration behaviour of facial branchiomotor neurons (FBMNs) in zebrafish embryonic hindbrain was studied using the DPA assay (Stockinger et al., 2011). Measurements on isolated cells revealed higher cohesion among FBMNs compared to cohesion among surrounding neuroepithelial cells (NCs). However, modulating NC cohesion by interfering with Cadherin-2 function severely affects FBMN migration *in vivo*, demonstrating that neuroepithelial cohesion guides FBMN migration, probably by restricting FBMN movement. In combination with high-resolution optical imaging, insight gained from *in vitro* adhesion force measurements thus serve to better explain mechanisms controlling *in vivo* cell migration in developing embryos.

An important advantage of DPA is that the accumulation of fluorescent fusion proteins involved in cell adhesion in the cell–cell

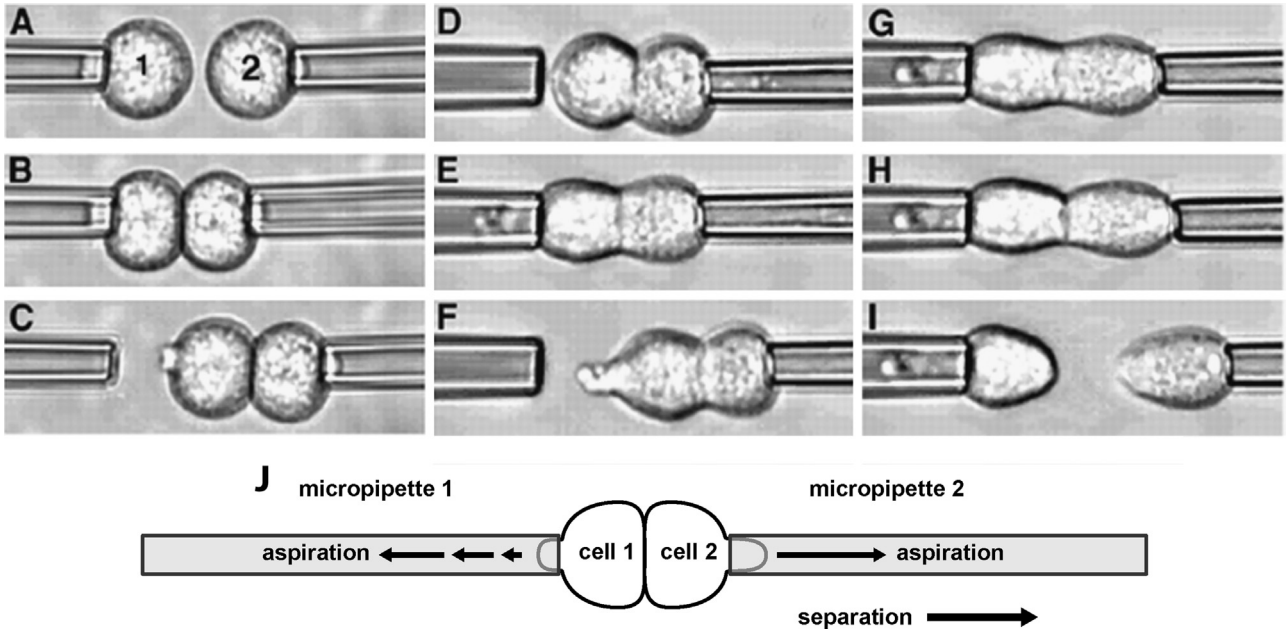

Fig. 3. Dual micropipette assay (A). Two cells, each held at the tip of a micropipette by weak aspiration, are brought into contact (B). Formation of cell–cell contact is tested by displacing the right micropipette (C). Subsequently, aspiration in the right pipette is increased to firmly hold the cell pair (D), while the aspiration in the other pipette is gradually increased until step by step displacement of the right pipette leads to cell separation (E–I). Reproduced with permission from Chu et al. (2004). (J) Schematic depiction of a DPA setup indicating the direction of the applied aspiration.

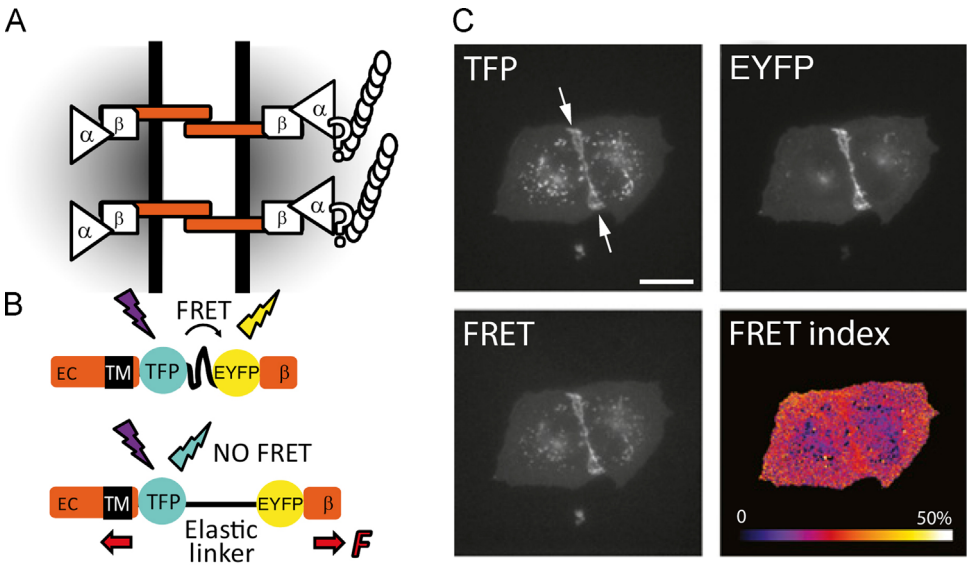

Fig. 4. Principle of the E-cadherin Förster resonance energy transfer (FRET)-based molecular tension sensor (EcadTSMod). (A) Working model for mechanotransduction through the E-cadherin/catenin complex. E-cadherin transmits mechanical tension between cells via transinteracting extracellular (EC) domains to the actin cytoskeleton through β-catenin, αE-catenin, and possibly other proteins. (B) The tension sensitive module (TSMod) consists of the mTFP/mEYFP FRET pair separated by an elastic linker $(GPGGA)_8$ derived from spider silk. The TSMod is inserted into the cytoplasmic domain of E-cadherin, where it can sense forces transmitted between the transmembrane domain (TM) and the β-catenin-binding domain (β). High and low FRET indices correspond to low and high tension, respectively. (C) Fluorescence imaging of two adherent MDCK cells expressing the EcadTSMod construct in the mTFP, mEYFP, and FRET (mTFP excitation; mEYFP emission) channels, and the corresponding map of the FRET index$=I_{FRET}/(I_{FRET}+I_{mTFP})$, where I is the fluorescence intensity of the subscript channel corrected for background and spectral bleed-through. Scale bar: 20 μm. Figure taken and modified with permission from Borghi et al. (2012).

zone can be easily studied by light microscopy and correlated with the separation force measurements (Chu et al., 2004; Maitre et al., 2012). Furthermore, the horizontal arrangement of the probed cell pair also permits determining cell shape changes during contact and after cell separation, which can provide unique insight into cortex tension at the cell–cell interface (Maitre et al., 2012). For instance, membranes at dissolved cell–cell contacts have been observed to display a rapid increase in curvature, indicating reduced cortex tension in these areas. Reduced cortex tension at cell–cell contacts helps to maximize the contact area between spherical cells and provides the driving force for cell–cell contact enlargement, while cadherin adhesion receptors function to mechanically link the cells (Maitre and Heisenberg, 2011).

Comparing SCFS and DPA

DPA measures separation forces between cells in suspension and this technique is therefore particularly well-suited to investigate adhesion between non-adherent cells, such as cells of the

immune system (Sung et al., 1986). In contrast, adherent cells first need to be removed from their physiological environment and brought into suspension. While suspended cells do not receive the structural, mechanical and biochemical cues of their native environment, their roughly spherical shape ensures reproducible contact conditions, and potential complications arising from complex matrix interactions are avoided (Chu et al., 2004). For instance, it is known that integrin-mediated interactions to the extracellular matrix can modulate cadherin activity (Martinez-Rico et al., 2010; Monier-Gavelle and Duband, 1997). Nevertheless, the absence of matrix and additional cell interactions should be taken into account when applying results from *in vitro* DPA measurements to explain cell behaviour in intact tissues.

In SCFS cells also need to be dissociated and suspended before attachment to a functionalized AFM cantilever, while the second cell must be attached to an adhesive substrate. The type of cantilever functionalization can have a significant impact on the scale of cell–cell adhesion forces (Friedrichs et al., 2010), indicating cross talk between the cell–surface and the cell–cell interactions. The requirement for cell immobilization also limits the maximal detectable separation force in SCFS, as only forces below the coupling strength of the cell to the force sensor (typically $\sim$30–40 pN) can be measured. As a result, SCFS measurements are commonly limited to the early phase of adhesion formation ($\leq$10 min) before cells have established their final adhesion strength. DPA is not limited in this regard and permits measuring even high separation forces ($\sim$200–400 nN) after cell contacts have fully matured after $\sim$60–90 min of contact (Angres et al., 1996; Chu et al., 2004, 2006). In any case, in single-cell assays cells can only be measured sequentially and the use of long contact times therefore severely limit the experimental throughput and extend the time necessary to collect statistically relevant data.

Determining accurate cell separation forces by DPA requires a series of micropipette retraction steps marked by a stepwise increase in aspiration strength. Therefore, adhesion sites in the cell doublet repeatedly experience tugging forces with increasing magnitude until successful cell–cell separation. However, cadherin contacts are mechanosensitive and repeated tugging has been shown to reinforce cadherin adhesion sites (Liu et al., 2010). So far, it is unknown whether failed cell–cell separation attempts in DPA promote contact enlargement and adhesion reinforcement. In contrast, in SCFS cell–cell separation is always achieved in a single step, avoiding the potential activation of mechanosensitive signalling pathways leading to adhesion reinforcement.

Aspiration in DPA also induces strong local cell membrane bending into the micropipette orifice, affecting cell cortex organization and possibly transmembrane protein density (Aimon et al., 2014). Strong aspiration can even induce membrane-cortex separation (Merkel et al., 2000; Rentsch and Keller, 2000). It is unknown how these aspiration-induced mechanical and biochemical changes affect cell–cell contacts forming at the opposite side of the cell. Optical microscopy during DPA frequently demonstrates pulling of long tethers out of the cell membrane during cell separation, which could affect the final adhesion force measurement. Similar membrane tethers are also often pulled from the cell surface during SCFS, but due to the vertical pulling direction in AFM these membrane tubes are more challenging to visualize (Krieg et al., 2008b). However, tether extension leaves plateau-like traces in AFM force-distance curves, which can be analysed quantitatively (Sun et al., 2005).

Förster resonance energy transfer (FRET)-based molecular tension sensors

Cadherins promote adhesion between neighboring cells but they are also involved in mechanotransduction via their cytoplasmic domain and interactions with catenin proteins and associated cytoskeletal regulatory and linker proteins. The E-cadherin/catenin complex has been furthermore demonstrated to act as a mechanical stress sensor and to modulate actomyosin contractility (Borghi et al., 2012; Ladoux et al., 2010; le Duc et al., 2010; Taguchi et al., 2011; Yonemura et al., 2010). Recently, Borghi et al. introduced an elegant method to visualize mechanical forces exerted at cadherin contacts by transforming them into optical signals using a FRET-based molecular tension sensor module (TSMod). The TSMod consists of the monomeric teal fluorescence protein (mTFP)/monomeric enhanced yellow fluorescence protein (mEYFP) FRET pair, connected via a 40 amino acid elastic $(GPGGA)_8$ domain derived from the spider silk protein flagelliform which acts as an entropic nanospring (Becker et al., 2003; Grashoff et al., 2010; Wang et al., 2011). The tension sensor module was introduced into the cytoplasmic domain of E-cadherin between the transmembrane and catenin-binding domain (Borghi et al., 2012). FRET is highly sensitive to the distance between the fluorophores and increased tension therefore leads to decreased FRET efficiency (Fig. 4). This method was established in Madin–Darby canine kidney (MDCK) cell culture, where the authors could demonstrate that the FRET efficiency of EcadTSMod at cell–cell contact sites was significantly decreased compared to soluble cytoplasmic TSMod or to an EcadTSMod deletion construct lacking the cytoplasmic domain of E-cadherin. This indicates that the EcadTSMod is under tension in cell–cell contacts and that this tension depends on the catenin-binding domain of E-cadherin. The authors calculated that the E-cadherin cytoplasmic domain is under 1 to 2 pN of constitutive load from the cytoskeleton, similar to vinculin in cell–matrix sites (Grashoff et al., 2010). Surprisingly, it was also shown that E-cadherin is under actomyosin-dependent tension even in regions of the plasma membrane containing no cell–cell adhesion sites, suggesting that the cadherin/catenin complex could act as a constitutive membrane anchor to the cortical actomyosin cytoskeleton (Borghi et al., 2012, Fig. 5). The authors further speculate that membrane attachment to the cortex by the cadherin–catenin complex could be relevant for single wound closure in *Drosophila* and plasma membrane blebbing during zebrafish epiboly (Abreu-Blanco et al., 2011; Schepis et al., 2012).

Another recent study demonstrates that FRET tensions sensors can also be employed to investigate developmentally-regulated cell–cell adhesion processes. Modulation of N-cadherin mediated cell–cell adhesion and tension is important for collective migration of *Xenopus* CNCC (Kuriyama et al., 2014), while recruitment of vinculin to cell–cell adhesion complexes is known to mediate mechanotransduction (Ishiyama et al., 2013; Miyake et al., 2006; Sumida et al., 2011; Taguchi et al., 2011; Yonemura et al., 2010). To analyze indirectly N-cadherin dependent tension at cell–cell contacts, the authors used a vinculin tension sensor (Grashoff et al., 2010). Blocking N-cadherin expression or inhibiting actomyosin contractility led to a release of junctional tension. Interestingly, inhibiting LPA signalling, which controls N-cadherin internalization, resulted in accumulation of N-cadherin at cell–cell contacts and increased tension trough vinculin. Enhanced N-cadherin mediated cell–cell adhesion could explain why CNCC are unable to disperse after depletion of LPA signalling, resulting in block of CNCC migration *in vivo*. Importantly, blocking N-cadherin expression, its homotypic binding activity or endocytosis rescues CNCC migration in LPA signalling deficient cells (Kuriyama et al., 2014). Thus, modulated cell–cell adhesion and tension is crucial for CNCC migration *in vivo*. When using the vinculin tension sensor one must however consider that vinculin localizes to both cell–cell contact sites and focal adhesions, cell–matrix sites at the cell–substrate interface. Therefore, in FRET experiments aimed at determining junctional tension, it is important to place the confocal imaging plane across the cell–cell contacts at the apical site of the cell, which avoids detecting vinculin-mediated tension at basal focal adhesion sites. Besides N-cadherin *Xenopus* CNCC also express the classical cadherin-11, which was shown to promote CNCC migration (Becker et al., 2013; Borchers et al., 2001; Kashef et al., 2009).

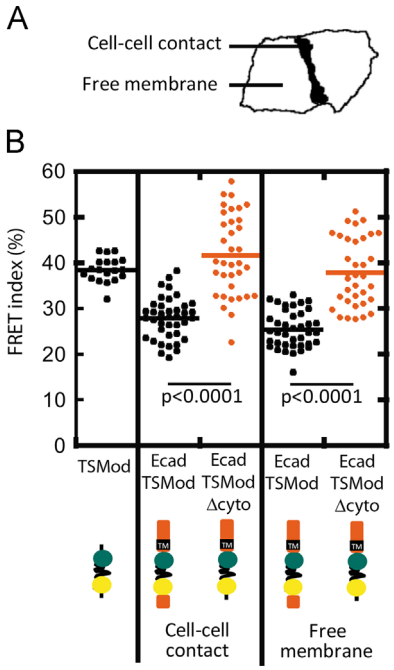

Fig. 5. Statistical analysis of FRET index measurements. (A) FRET index measured at cell–cell contacts and at free cell membranes reveals that EcadTSMod is under constitutive tension. (B) FRET index for TSMod in the cytoplasm, EcadTSMod and EcadTSModΔcyto at cell–cell contacts, and EcadTSMod and EcadTSModΔcyto at free membranes in MDCK cells. EcadTSMod yields a lower FRET index than either EcadTSModΔcyto or cytoplasmic TSMod, indicating constitutive tension of membrane-inserted E-cadherin. *P* values are calculated using a two-tailed Mann–Whitney test. Figure taken with permission from Borghi et al. (2012).

Introducing the TSMod into the cytoplasmic domain of cadherin-11 could therefore be a novel tool to examine a possible role of cadherin-11 in mediating cell tension during CNCC migration.

Outlook

The introduction of sensitive and quantitative single-cell assays has provided a wealth of information regarding cell–cell adhesion forces. Both DPA and SCFS probe receptors presented in their physiological environment on the surface of living cells. This provides an important advantage, because associated intracellular proteins mediating linkage to the cytoskeleton may modulate the receptor adhesion strength. The effects of inhibitors or stimulators of adhesion can also be investigated directly in the context of a living cell. Nevertheless, there is still little knowledge regarding the effective mechanical and adhesive forces acting in developing tissues, and correlating results from *in vitro* assays with cell behavior *in vivo* remains an ongoing challenge. Laser ablation experiments can detect tension within intact tissues, but quantitatively analyzing tension is difficult because the exact mechanical properties of the severed tissue are usually unknown. Furthermore, although intercellular adhesion contributes to the distribution of tissue tension, ablation experiments provide no quantitative read-out of cell–cell adhesion strength. Recently, an elegant alternative method has been presented to quantify adhesion-dependent mechanical forces directly in living tissues based on the deformation of fluorescent, cell-sized oil microdroplets with defined mechanical properties and surface functionalization (Campas et al., 2014). In this approach, cell membrane-impermeable fluorocarbon oil droplets are stabilized by a biocompatible, amphiphilic surfactant carrying a polyethylene glycol spacer coupled to biotin. Using streptavidin as a linker, other biotinylated ligands, such as the RGD peptide (a ligand for integrin receptors) or anti-E-cadherin antibodies, can then be easily coupled to the droplet surface. Furthermore, using fluorescently-labeled streptavidin permits microscopic observation of the droplet shape. In the absence of external forces oil microdroplets will have a spherical shape in aqueous medium as a result of isotropic hydrostatic pressure. When the microdroplets are injected into the intercellular space of tissues, the degree of droplet deformation therefore visualizes local anisotropic tissue stress. For a quantitative analysis of tissue stress, the shapes of individual droplets are first reconstructed from confocal image stacks. The local deviation from a spherical shape then provides a measure for the pulling or pushing stresses cells have applied on every point on the droplet surface. By adding fluorocarbon-hydrocarbon co-surfactants, the interfacial tension of the fluorocarbon droplets can be tailored to match the effective stress levels applied by different cell types. In this pioneering study, this technique has been applied to highlight different scales of tissue stress between embryonic tooth mesenchyme and developing mandibles, but it should be possible to readily apply these technique to other tissue models in future studies.

Single-cell adhesion measurements often reveal large cell-to-cell variations. Given the well-controlled cell–cell contact conditions during DPA and SCFS, these differences are unlikely to originate from vastly different contact areas or mechanics between the probe cells, but some variation may be linked to different cell cycle stages. However, cell populations also display adhesion variability which is cell–cell-independent and which correlates with a natural variation of receptor expression levels between individual cells (Dao et al., 2012). Compared to single-cell measurements to adhesive substrates, testing cell pairs introduces additional variability and requires a larger number of test repetitions to establish statistically significant differences between experimental conditions. Nevertheless, the additional molecular insight offered by single-cell techniques offsets some of their disadvantages, such as the comparatively low experimental throughput. Taken together, the ongoing development and improvement of quantitative single-cell methods is providing exciting novel insight into the dynamic regulation of cell–cell adhesion during the formation of multicellular organisms. Since the dysregulation of adhesion receptors and their signaling function also lead to a broad variety of pathological defects, these tools also improve our understanding how changes in cell adhesion can contribute to different diseases.

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